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Identification of plasma membrane associated mature β-hexosaminidase A, active towards GM2 ganglioside, in human fibroblasts

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Abstract Mature β-hexosaminidase A has been found associated to the external leaflet of plasma membrane of cultured fibroblasts. The plasma membrane association of β-hexosaminidase A has been directly determined by cell surface biotinylation followed by affinity chromatography purification of the biotinylated proteins, and by immunocytochemistry. The immunological and biochemical characterization of biotinylated β-hexosaminidase A revealed that the plasma membrane associated enzyme is fully processed, suggesting its lysosomal origin.

Keywords: β-Hexosaminidase; Lysosomal enzyme; Plasma membrane

1. Introduction

Plasma membrane glycoconjugates show changes during development and neoplastic transformation [1,2] suggesting a specific role in cell–cell recognition. Glycosyl epitope changes include many different residues, such as GlcNAc, GalNAc in N-linked structures or sialyl residues in either protein N-linked, O-linked or lipid linked structures, so that cell surface glycohydrolases could be one of the natural candidate for plasma membrane glycosylation modification.

For this reason, although in the past little attention was paid to glycohydrolases acting on cellular compartment different from lysosomes, there is now increasing interest on plasma membrane associated glycohydrolases because on this compartment they should be involved, together with glycosyltransferases, in GSL oligosaccharide modification processes regulating cell-to-cell and/or cell–environment interactions in both physiological and pathological mechanisms [3–5].

β-Hexosaminidase (E.C.3.2.1.52) is an acidic glycohydrolase that cleaves terminal β-linked GlcNAc or GalNAc residues from oligosaccharides, glycolipids, glycoproteins and glycosaminoglycans. Two major lysosomal isoenzymes exist in human tissues which are the products of the assembly of two subunits, α and β, encoded by two closely related genes [6,7]. The two isoenzymes Hex A (αβ) and Hex B (ββ), differ in their substrate specificity. Both isoenzymes are able to hydrolyse several natural and artificial substrates, but only Hex A, in combination with a specific activator protein [8], can hydrolyse GM2 ganglioside. β-Hexosaminidase precursors α and β chains (pro-chains) are sorted out from *trans*-Golgi-network (TGN) by mannose-6-phosphate receptors (MPR) after de novo synthesis, and transported to lysosomes, where the two subunits undergo final processing to produce the complex polypeptide structure of the mature enzyme [9]. A small part of neo-synthetized β-hexosaminidase pro-chains escapes from the direct transport to endosome/lysosomes and recycles via cell-surface in a MPR-bound form [10].

In the present work we show the first evidence for the presence of a fully processed β-hexosaminidase A (Hex A) associated to the external leaflet of the plasma membrane of human fibroblasts, with a direct approach by in vivo cell surface biotinylation. Moreover we demonstrate its enzymatic activity towards the natural substrate GM2 ganglioside.

2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute 1640 medium, Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), Trypsin, Penicillin/Streptomicin were from Biochrom KG Seromed; 4-methylumbelliferyl-β-*N*-acetylglucosaminide-6-sulphate (MUGS) was from Toronto Research Chemicals Inc. 4-Methylumbelliferyl-β-*N*-acetylglucosaminide (MUG), 4-methylumbelliferone, the detergents NP40 and Triton X-100; protease inhibitor cocktail for mammalian cell extracts, monoclonal mouse anti-goat IgG HRP conjugate were from Sigma-Aldrich Fine Chemicals Co. Alexa Fluor 488 rabbit anti-goat IgG and Alexa Fluor 546 rabbit anti-mouse IgG were from Molecular Probes, Inc. Monoclonal mouse anti-LAMP-2 was from Santa Cruz Biotechnology, Inc. Hybond-C Extra nitrocellulose, ECL Western Blotting detection reagents and Sepharose-G-protein slurry were

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from Amersham Biosciences. Bovine serum albumin and Bio-Rad protein assay reagent were from Bio-Rad Laboratories. Sulfosuccimidyl-6-(biotinamido)hexanoate (EZ-link™ Sulfo-NHS-LC-Biotin), ImmunoPure Monomeric Avidin Kit and HRP-streptavidin were from Pierce, Centricon YM-10 were from Amicon, Millipore. All other reagents were of analytical grade. Gangliosides GM2 and GM3 were prepared, characterized and tritium labelled at position 3 of *erythro* C18-sphingosine as described [11–14]. The recombinant human GM2 activator protein, GM2AP, was produced in *Escherichia coli* [15].

2.2. Cell cultures

Human fibroblasts were cultured in DMEM medium containing 10% (v/v) heat inactivated FBS, penicillin 100 IU/ml/Streptomycin 100 μg/ml. Cell viability determined by Trypan blue method, under the differential experimental conditions was about 95%.

2.3. Immunocytochemical labelling of plasma membrane β-hexosaminidase

Normal human fibroblasts were plated onto glass coverslips and grown for 24 h before labelling. Cells were washed with phosphate-buffered saline (PBS) buffer and then fixed with 4% paraformaldehyde/PBS for 10 min at RT. After further washings cells were blocked and permeabilized in 0.2% Triton X-100/PBS containing 10% (w/v) bovine serum albumin (blocking solution) for 30 min at RT. Cells were then treated with primary goat anti-human placental Hex A [16] and mouse anti-LAMP-2 antibodies diluted in blocking solution. After washings cells were incubated with Alexa Fluor 488 anti-goat IgG and Alexa Fluor 546 anti-mouse IgG antibodies diluted in blocking solution. For the restrictive plasma membrane staining, cells were incubated exclusively with the antibody anti Hex A in the same conditions as above, but without Triton X-100. Fluorescence microscopy was carried out using a Nikon TE2000 microscope. Double labelling was also examined by a confocal scanning microscope Nikon PCM2000.

2.4. β-Hexosaminidase assays

β-Hexosaminidase activity was measured as previously described [17] on artificial substrates MUG and MUGS. Fluorescence was measured on a Perkin–Elmer LS B50 fluorimeter (excitation, 360 nm; emission, 446 nm). Optimum pH for β-hexosaminidase activity was determined using the substrate MUG in 0.1 M citric acid/0.2 M disodium phosphate buffers at different pH values ranging from pH 3.5 to 7.5. One unit (U) is the amount of enzyme that hydrolyses 1 μmol of substrate/min at 37 °C. Protein content was determined [18] using bovine serum albumin as a standard. Specific activity was expressed as U/mg of protein. β-Hexosaminidase activity on natural substrate was determined using [^{3}H] GM2. The assay was performed using about 30 μg homogenate cell proteins or using purified plasma membrane proteins corresponding to about one mU of enzyme, as determined on MUG, in a final volume of 50 μl of 10 mM, pH 4.2, citrate buffer, containing 0.1% bovine albumin, 25 μg GM2AP, 13 μg of sodium taurodeoxycholate and 10.000 dpm of GM2 diluted in 3 μg cold GM2. The enzyme reaction mixture was maintained at 37 °C under continuous vortexing for 12 h. The reaction mixture was mixed with 3 volumes of tetrahydrofurane, the mixture was then centrifuged, the clear solution dried and the residue resuspended into a few μl of chloroform/methanol 2:1 (v/v). The enzyme reaction mixtures were analyzed by HPTLC followed by radioimaging and quantitative detection of the separated GM2 and GM3.

2.5. Biotinylation of cell surface proteins

Cells were washed three times with ice-cold 8 mM Na_2HPO_4/1.5 mM KH_2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.5 (Dulbecco's PBS) each flask of fibroblasts was incubated, under gentle shaking, for 30 min at 4 °C in 4 ml solution of EZ-Link Sulfo-NHS-LC-Biotin, dissolved in PBS at the concentration of 0.5 mg/ml [19]. The reaction was terminated by adding 4 ml of 100 mM Tris/HCl, pH 7.5. Cells were then washed three times with PBS, and treated with the lysis buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 0.5% Triton X-100 and protease inhibitor cocktail). After a 30 min incubation at room temperature, cell homogenate was sonicated (four sonications, 15 s each) and recovered and used for affinity chromatography.

2.6. Affinity chromatography

Biotinylated proteins from fibroblasts were purified by monomeric avidin affinity column chromatography, according to the manufacturer instructions. Briefly, the column (2 ml volume) was washed with 2 mM biotin in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 (PBS) to block any non-reversible biotin binding site contained in avidin resin. The loosely bound biotin was removed with 0.1 M glycine pH 2.8; the column was then equilibrated with PBS. Cell homogenate was loaded, then the column was washed with PBS containing 0.1% Triton X-100 until all unbound protein had been washed off and the A_{280} returned to baseline. Retained (biotinylated) protein was then eluted using 5 mM D-biotin in PBS containing 0.1% Triton X-100. One ml fractions were collected. Unretained and biotinylated proteins were pooled separately and used for further characterizations.

2.7. Western blotting analysis of β-hexosaminidase polypeptides

Samples containing the same units of β-hexosaminidase were precipitated with 10% TCA and the resulting pellets subjected to 10% SDS–PAGE under reducing conditions according to Laemmli [20]. Proteins were transferred to nitrocellulose, and the α and β peptides of β-hexosaminidase recognized by immunoblotting using respectively an antiserum specific for α-subunit, raised to a mixture of synthetic peptides belonging to the sequence of mature human enzyme, and an anti-β-subunit raised to Hex B purified from human placenta, followed by enhanced chemiluminescence detection.

2.8. Immunoprecipitation studies

Biotinylated fibroblasts were detached by scraping the cells in 1% EDTA PBS. Cells were recovered by centrifugation and subjected to three washes in ice-cold PBS. Fibroblasts from a 75 cm^2 tissue culture flask were resuspended in 250 μl of lysis buffer and extracted as above described. Cell homogenate, precleared with Sepharose-protein-G slurry by rotation for 1 h at 4 °C, was immunoprecipitated using the antiserum specific for β-subunit, previously bound to Sepharose-G-protein slurry by rotation for 2.5 h at 4 °C. After an overnight incubation at 4 °C and centrifugation, immunocomplexes bound to Sepharose-protein-G slurry were extensively washed [21] and finally solubilized in Laemmli sample buffer [20]. The same procedure was applied to not biotinylated fibroblasts. Biotinylated cell homogenate treated as above but without antibody was used as negative control. Analysis of the immunocomplexes was performed by Western blotting and bands detected by incubating the filter for 1 h with HRP-streptavidin diluted 1:500 in PBS, pH 7.4, and ECL detection reagent.

3. Results and discussion

Cell surface protein biotinylation, in vivo, followed by avidin affinity chromatography represents a good tool to purify plasma membrane (PM) associated proteins. The procedure allowed us to characterize a PM associated β-hexosaminidase avoiding contamination of lysosomal activity and "in transit" isoenzymes during secretion.

The large majority of total cell β-hexosaminidase activity towards artificial substrates, MUG (hydrolysed by both α- and β-subunits) and MUGS (hydrolysed exclusively by α-subunit), was not retained by the affinity column. Nevertheless, a minor enzyme activity was retained by the column and then recovered as biotinylated fraction. From four different experiments, this activity represented 3–5% of total activity loaded on the column and was displayed towards both substrates, MUG and MUGS.

An additional evidence for the presence of β-hexosaminidase on the outer leaflet of PM was achieved by immunocytochemical labelling of cells using a polyclonal antiserum, raised against human placental Hex A, followed by conventional (Fig. 1, panel a) and confocal (Fig. 1, panel b) fluorescence microscopy. To specifically visualize the lysosomal compartment, cells were also immunostained with the anti-LAMP-2 antibody.

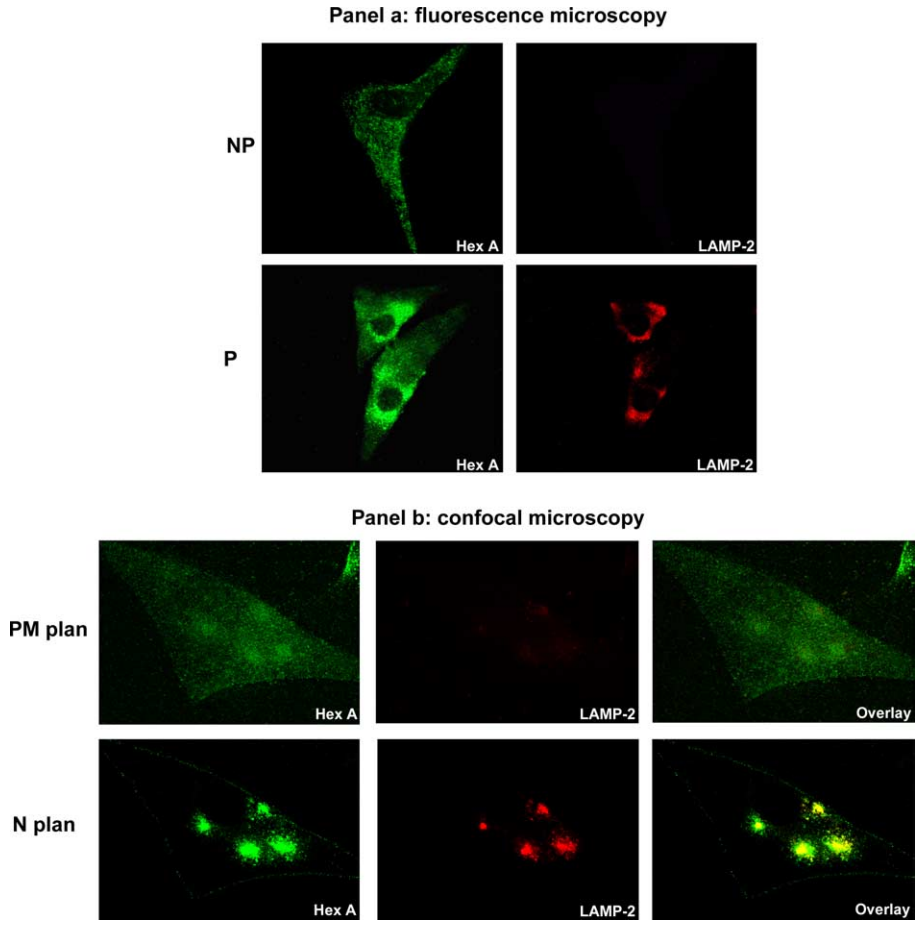

Fig. 1. Immunofluorescence detection of cell surface Hex A in human fibroblasts. *Panel a*. Fluorescence microscopy. NP: double labelling in non permeabilized cells (NP) with goat anti-human placental Hex A (Hex A) and mouse anti-human LAMP-2 (LAMP-2), antibodies. P: double labelling in permeabilized cells (P) with goat anti-human placental Hex A antibody (P, Hex A) and mouse anti-human LAMP-2 (P, LAMP-2). Fluorescence was analyzed using a Nikon TE2000 microscope. Magnification, 40×. *Panel b*. Confocal microscopy. Double labelling in permeabilized cells with same antibodies as described in panel a. PM plan: Hex distribution (Hex A) is shown in green, LAMP-2 distribution (LAMP-2) in red and colocalization of both proteins in yellow (overlay). N plan: Hex distribution (Hex A) is shown in green, LAMP-2 distribution (LAMP-2) in red and colocalization of both proteins in yellow (overlay). Fluorescence was analyzed using a confocal scanning microscope Nikon PCM2000. Magnification, 60×. Section thickness was 0.8 μm.

When the Hex A labelling was performed on non-permeabilized cells we had fluorescence staining on the PM (Fig. 1, panel a). Under similar conditions no staining was detected for LAMP-2. Labelling performed on permeabilized cells clearly detected the lysosomal enzyme and a minor quantity associated to PM. In an experiment we pre-incubated cells 6 hr with cycloheximide, an inhibitor of protein biosynthesis. Under this experimental conditions the PM associated labelling was not inhibited or modified (data not shown), indicating that PM associated β-hexosaminidase has an half life longer than 6 h. This result would exclude that cell surface β-hexosaminidase staining is due to MPR trafficking toward the membrane. Confocal microscopy results (Fig. 1, panel b) confirmed the presence of hexosaminidase on PM. Plasma membrane plan visualization showed a diffuse specific Hex A staining, while only traces of LAMP-2 were visible (this last observation is in agreement with that previous made by Eskelinen et al. [22]). As expected, on the nucleus plan, intracellular LAMP-2 staining completely merged with Hex A immunolabelling, this highlighting the large lysosomal cell distribution of the enzyme.

Biotinylated proteins from human fibroblasts purified by affinity chromatography were analyzed by Western blotting using the two polyclonal antibodies specific for the α- and β-subunit, respectively. Fig. 2 shows that both PM associated and lysosomal α- and β-subunits displayed the same proteolitic processing, indicating that the PM associated enzyme is in its mature form. In fact, the anti α-subunit antibody recognized in both the plasma membrane and soluble fraction a protein displaying molecular mass of 54 kDa, while the anti β-subunit antibody recognized in both cases a protein of 30 kDa [16]. These two values correspond to the fully processed subunits. Immunoprecipitation experiments performed with anti β-subunit antibody on the total homogenate from biotinylated fibroblasts, showed that both α- and β-subunit, respectively, were present in the immunoprecipitate as biotinylated proteins (Fig. 3), an evidence that mature Hex A isoenzyme (an α–β dimer) is present on the cell surface.

PM associated β-hexosaminidase obtained by affinity chromatography displayed optimal pH curves for activity towards both MUG and MUGS substrates close to those of soluble (fraction not-retained by the affinity column) counterpart, with

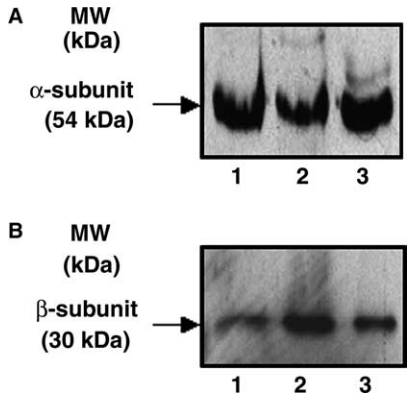

Fig. 2. Western blotting analysis of cell homogenate and biotinylated membrane-associated β-hexosaminidase. After cell surface protein biotinylation, biotinylated proteins were separated by avidin affinity chromatography as reported in the experimental section, subjected to SDS–PAGE and analyzed by Western blotting. (A) Immunostaining with anti α-subunit antibody; (B) immunostaining with β-subunit antibody. Lane 1, cell homogenate; lane 2, affinity chromatography unretained intracellular fraction; lane 3, affinity chromatography retained biotinylated membrane-associated fraction.

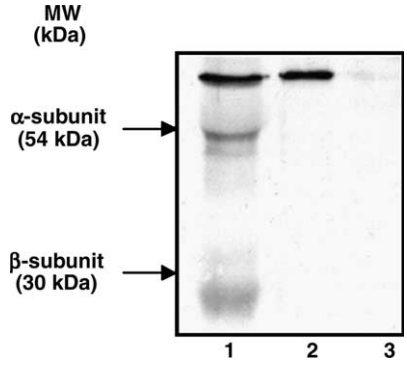

Fig. 3. Western blotting analysis of immunoprecipitated plasma membrane associated Hex A. After cell surface biotinylation and cell homogenization, proteins were immunoprecipitated with an anti β-subunit polyclonal antibody. The immunoprecipitate was subjected to SDS–PAGE and successive Western blotting analysis using HRP-conjugated streptavidin. Lane 1, anti β-subunit immunoprecipitate from biotinylated cells; lane 2, negative control; lane 3, anti β-subunit immunoprecipitate from non biotinylated cells.

a maximum in both cases at 4.5 (Fig. 4). At a pH above 7.0 about 10% of the maximum enzyme activity is maintained and this is true for both lysosomal and PM-associated hexosaminidases. Presence on the plasma membrane of glycohydrolases displaying an acidic optimum pH, is not a novelty. It has already been demonstrated that human plasma-membrane-associated sialidase NEU3 displays an acidic optimum pH [23,24]. Acidic pH can be obtained in specific zones of the external leaflet of the plasma membrane by plasma membrane associated proteins such as Na^+/H^+ antiporter [25,26] or following segregation of acidic sialocompounds. It is well known that on the plasma membrane some gangliosides can be highly enriched, together with specialized proteins, in membrane lipid domains [27].

Biotinylated β-hexosaminidase from fibroblasts showed enzymatic activity towards the natural substrate GM2 ganglioside in the presence of GM2 activator protein. Fig. 5 shows

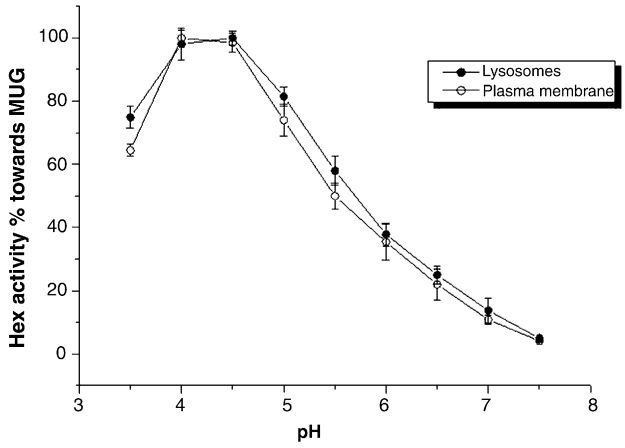

Fig. 4. pH Optimum curves of lysosomal and plasma-membrane associated β-hexosaminidase. Hex A activity towards MUG was determined in the pH range 3.5–7.5 in 0.1 M citric acid/0.2 disodium phosphate buffer using aliquots of biotinylated membrane-associated Hex A (○) and unretained intracellular Hex A (●). Results were expressed as percentage of the maximum value, corresponding to optimum pH, and are the mean of three independent experiments.

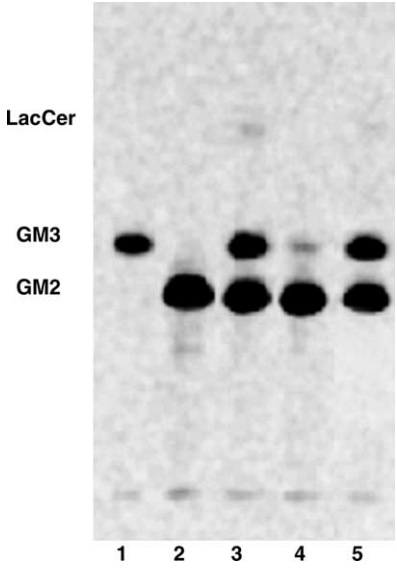

Fig. 5. Cell homogenate and plasma-membrane associated β-hexosaminidase activity on GM2 ganglioside The assay was performed in the presence of the GM2 activator protein as reported in the experimental section, after cell surface protein biotinylation and avidin affinity chromatography as reported in the experimental section. The total enzyme reaction mixture was separated by TLC and radioactive lipids were detected by digital autoradiography; 200–400 dpm were applied on a 4 mm line; time of acquisition: 24 h. Lane 1, standard GM3; lane 2, negative control; lane 3, fibroblast cell homogenate; lane 4, affinity chromatography retained biotinylated membrane-associated fraction; lane 5, affinity chromatography non-retained fraction.

that, in an in vitro assay, the GM2 ganglioside, containing tritiated sphingosine, was transformed in GM3. This result suggests the perspective of a potential functional role of PM β-hexosaminidase in human fibroblasts.

Like other lysosomal enzymes, after de novo synthesis, β-hexosaminidase is transported from TGN to lysosomes, where undergoes the final proteolytic processing [9]. The presence of

fully processed α- and β-subunits on PM indicates that the enzyme is delivered to PM from lysosomes. We can exclude that the PM β-hexosaminidase is associated to the cell surface by MPR interaction, because it is known that the lysosomal enzymes loose the phosphate marker when they reach lysosomes [28]. Fusion events of lysosomes with the PM, in response to a rise of cytosolic Ca^{2+}, have been described as a process for surface damage repairing [29,30].

Evidence supporting the existence of alternate targeting mechanisms for lysosomes have been reported for a variety of cell types including fibroblasts [31,32]. Moreover a number of lysosomal enzymes have been reported to associate with lysosomal membranes by a M6P-independent mechanism [33–35].

Our suggestion about the existence of a direct exchange of β-hexosaminidase between lysosomes and PM, raises the issue to explain how this enzyme can be both a lysosomal soluble and membrane associated protein. To gain information about this, we performed computer analysis along the primary structure of β-hexosaminidase subunits [36]. Results did not show any multiple stretches of hydrophobic aminoacids and did not suggest the possible addition of a glycosylphosphatidylinositol anchor that could justify the association of Hex A to PM. Nevertheless, we cannot exclude post transductional modifications able to introduce into the protein small hydrophobic changes that allow interactions with the membrane hydrophobic layer.

Evidences for membrane associated β-hexosaminidase activity has been provided in *Drosophila melanogaster* and in *Ascidia* sperm, and a role for the enzyme in sperm-egg recognition has been suggested [37–41]. In humans evidences are limited to demonstration of hexosaminidase activity, detected only by synthetic substrates, in PM enriched subcellular fraction of peripheral blood lymphocytes and monocytes of multiple sclerosis patients, or of cancerous cells [42–44].

Our results, demonstrating the association to the extracellular leaflet of plasma membrane of a mature β-hexosaminidase A, in combination with the information on other plasma membrane associated glycohydrolases [5] is very attractive. The fact that the enzyme is active toward gangliosides adds specific interest to our information and new perspective for the biological meaning of the presence of the enzyme at the cell surface. In previous studies it has been demonstrated that plasma membrane associated sialidase is capable to hydrolyse gangliosides belonging to neighboring cells [5], thus determining the composition on the interacting surface. Gangliosides are highly enriched in membrane lipid domains where their concentration can participate to determine the correct acidic pH necessary for the maximum enzyme hydrolysis. A specific role of gangliosides to determine acidic conditions necessary to biological processes was presented in the past [45,46]. This sialidase hydrolyses the ganglioside Neu5Ac–Gal linkages quite rapidly. Nevertheless, when the sialic acid belongs to the trisaccharide sequence GalNAc–(Neu5Ac–)Gal, which is known to be very rigid due to the strong interactions occurring between the sialic acid lateral chain and the GalNAc residue [47], it cannot be released by the enzyme. Thus, the plasma membrane associated β-hexosaminidase can remove the GalNAc residue preparing the substrate for a further degradation.

According to this the availability of a pool of glycohydrolases associated to the plasma membrane allows structural changes of glycosphingolipids and changes of the organization of membrane lipid domains in which gangliosides are highly enriched. Membrane lipid domains segregate several proteins belonging to the signalling processes and the glycolipid oligosaccharide modifications could be instrumental to regulate cell-to-cell and/or cell–environment interactions in both physiological and pathological mechanisms.

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